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(54) Title: GLYCYL-HYSTIDYLE-LYSINE (GHL) DERIVATIVES (57) Abstract The invention refers to derivatives of the peptide Gly-His-Lys (GHL) having cytoestimulant and cytoprotective activity, to their therapeutic use and to pharmaceutical compositions containing them. The derivatives of the invention are surprisingly resistant to hydrolytic enzymes in addition to increase therapeutic activity.		

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GLYCYL-HISTIDYL-LYSINE (GHK) DERIVATIVES

The present invention refers to Gly-His-Lys (GHL) peptide derivatives having cytostimulant and cytoprotective activity, to their therapeutic use as well as to pharmaceutical compositions containing them.

5 The derivatives of the invention, together with improved therapeutic activities, are endowed with surprising resistance to hydrolytic enzymes.

Many methods are known in medicine for wound management, including particular devices (from the anti-infective medicated plaster to the synthetic skin
10 substitutes for burn patients) ointments, gels or other formulations for topical application containing for instance zinc derivatives.

Recently, as a consequence of a deeper knowledge
15 of the tissue repair mechanism and of the development of recombinant DNA techniques, different active principles have been prepared and studied, in order to promote and/or normalize the healing process and to heal tissue damages not otherwise curable (such as, for
20 instance, decubitus or diabetic ulcers) and to prevent the formation of scars and cheloids:

- growth factors such as: bFGF, basic Fibroblast Growth Factor; EGF, epidermal growth factor; GHL (also known as GHK), Plasma Copper-Binding Growth
25 Factor, etc.;
- components of the extracellular matrix such as fibronectin, collagens, etc..

However, a number of reasons make the practical

use of these derivatives difficult. They comprise the availability of the substance, the risk of transmission of viral or bacterial diseases, due to their origin, and, above all, their instability to enzymatic systems (such as proteases and peptidases: both in vitro and in vivo), characterising most proteins, glycoproteins or peptide substances and providing the major obstacle to their use as drugs, particularly in the pathologies in which the protease activity is increased, such as in inflammatory conditions, both systemic and local.

There is therefore the need for more stable active principles in the most different therapeutic fields.

In the specific case of healing, attempts to this aim on complex proteins such as EGF, bFGF and collagens - obtained by recombinant DNA or extraction - turn out to be difficult and the few known examples are based on the use of topical galenical forms containing both inhibitors of serine protease and, as for EGF, competitive substrates such as collagen (see for instance K. Okumura et Al. - Pharm. Res., 7, 1289, 1990).

As far as simple peptide molecules, such as Arg-Gly-Asp (RGD: known as the cellular adhesion site of fibronectin, laminin and of other proteins of the extra-cellular matrix) and Plasma Copper-Binding Growth Factor (GHL or GHK: tripeptide Gly-His-Lys) are concerned, the stabilization strategy used until now is based on the modification of the N- or C-ends (or of equivalent groups of the amino acid side-chains): introduction, with ester- or amide- like bonds, of suitable residues on said ends, leaving the peptide

backbone unchanged. These changes induce a certain resistance to protease degradation, but the half-life of the products is subjected to the local presence of different enzymatic systems such as esterases, more or less enhanced by the pathology of the interested tissue and able to cleave the introduced group (exposing therefore the resulting peptide to the natural demolition pathway).

In this respect, Pierschbacher M. (WO 90/06767, La Jolla Cancer Res.Found.) proposes polypeptide polymers of Arg-Gly-Asp (RGD), conjugated with biodegradable polymeric matrices, such as jaluronic acid, chondroitin sulphate, heparan sulphate, etc..

Pickart L.R. (US Patent 4,665,054 of 12.05.1985, applicant Biohead Inc.) discloses the healing activity of the copper complexes of glycyl-histidyl-lysine (GHL) peptide derivatives having the general formula: glycyl-histidyl-lysyl-COOR (RO=residues of alkyl or aryl alcohols; $-NH_2$ group) and claims their utility in the facilitation of the healing process and in the inhibition of thromboxane production by platelets. In comparison to the natural peptide, the said derivatives have an higher stability to hydrolysis by carboxypeptidases, i.e. the enzymes hydrolyzing the molecule starting from the carboxy-terminus (C-terminal).

In fact, the following half-life times (approximate and in excess) with respect to these enzymes can be derived from the data reported in said US patent: natural GHL < 1'; GHL-CONH₂ < 2'; GHLCOOMe < 1.5'; GHL-COOCH₂C₆H₅ > 8'-10'.

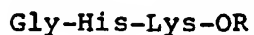
It has now been surprisingly found that the derivatives of the GHL peptide (and of the corresponding copper complex) according to this invention impart a general stability increase to the peptide (and to the corresponding copper complex) both with respect to the carboxy- and amino- peptidases and to the esterases.

The present invention provides a more favourable solution to the known problem in the stabilization of the GHL peptide. The derivatives of the invention provide an improvement to the applicative therapeutic fields (wound healing, ulcers and tissue damages of different etiology) and to cosmetic applications (increase of the subcutaneous fat; decrease of wrinkles and telangiectasia conditions; stimulation of hair growth) since, differently from the known derivatives disclosed by L.R. Pickart, they exhibit an higher and unexpected resistance to hydrolytic agents such as carboxypeptidases, aminopeptidases and esterases (the latter being able to hydrolyse the ester bond between GHL and an alcohol or amide residue, therefore exposing the resulting GHL to rapid subsequent degradation).

These products maintain the capacity of complexing the Cu ion and show biological activities higher than those of the natural derivative, both in terms of specific activity (defined as activity per weight unit) and in terms of prolonged action in time (both connected to the increased resistance to enzymatic hydrolysis).

The derivatives of the invention have the

following general formula:



(I)

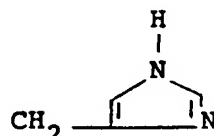
wherein:

Gly is one of the following residues:

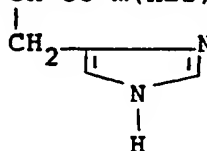
- 5 - glycine;
 - sarcosine;
 - group of formula $\text{NH}_2\text{-CH}_2\text{NH-}$, [gem(Gly)];

His is one of the following residues:

- 10 - L-histidine;
 - D-histidine;

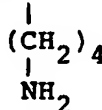


- group of formula HN-CH-NH , [gem(His)];
 15 - group of formula -CO-CH-CO m(His) ;



Lys is one of the following residues:

- 20 - L-lysine;
 - D-lysine;
 - group of formula -CO-CH-CO-(m-Lys)



- 25 R is hydrogen, straight or branched $\text{C}_1\text{-C}_{14}$ alkyl,
 $\text{C}_6\text{-C}_{14}$ aryl or aralkyl residue, with the proviso that
 Gly, His and Lys cannot be contemporaneously the
 natural amino acids glycine, L-histidine and L-lysine.

- 30 The invention also comprises the pharmaceutically
 acceptable salts and the copper complexes of the
 compound I.

Preferred compounds of formula I are those wherein:

- 1 - His or Lys is a residue of the corresponding D aminoacid and R is H;
- 5 2 - His or Lys is a residue of the corresponding D aminoacid and R is different from H;
- 3 - His or Lys are both residue of the corresponding D aminoacids and R is H;
- 10 4 - His or Lys are both residue of the corresponding D aminoacids and R is different from H;
- 5 - Gly is the sarcosine residue whereas His, Lys and R are as defined in any one of the above points 1-4;
- 15 6 - Gly is a gem-diaminal residue as above defined [gem(Gly)] whereas one of the His and Lys residue is a residue m(His) or m(Lys) as above defined whereas the other is a residue of the L or D series and R is hydrogen;
- 20 7 - Gly is a gem-diaminal residue as above defined [gem(Gly)] whereas one of the His and Lys residue is a residue m(His) or m(Lys) as above defined whereas the other is a residue of the L or D series and R is different from hydrogen;
- 25 8 - Gly is glycine, His is gem-His and Lys is m(Lys) as above defined and R is hydrogen;
- 9 - Gly is glycine, His is gem-His and Lys is m(Lys) as above defined and R is different from hydrogen.

With reference to the previously reported biological properties of the derivatives of the
30 invention, these may be used as therapeutic agents in pathological forms asking for a cytoprotective and/or

cytostimulating activity.

Particularly, the compounds of formula I are conveniently used for the preparation of a drug useful in treating ulcers, scars, tissue damages of different
5 kind and more generally of drugs for the treatment of autoimmune disease.

The compounds of the invention are formulated in suitable pharmaceutical compositions alone or in combination or with other useful active
10 principles.

The dosages will be determined by the physician and will anyhow depend on the pathology to be treated, age, weight and conditions of the patient. Examples of pharmaceutical compositions are topical forms such as
15 creams, ointments, gels, aspersion powders, medicated plasters, controlled release topical forms, local injection (e.g. intraarticular); systemic forms, such as injectable, or oral forms such as tablets, capsules or other conventionally known forms. The compositions
20 of the invention may be prepared by usual methods, such as those disclosed in Remington's Pharmaceutical Sciences Handbook, Mack Pub. Co., NY, USA.

The following examples further illustrate the invention.

25 The used reagents, chemicals and standards are usually available from commercial sources. The disclosed solid-phase synthetic methods for the derivatives containing either D or L aminoacids are those usually used in the peptide synthesis but they
30 should not be intended to limit the different synthetic possibilities which can be used according to the

available knowledges, such as, for instance, the synthesis in homogeneous phase, used herein for the derivatives esterified at the C-terminus.

5 The preparation of the retro-inverted derivatives containing also aminoacids of the L series relies on methods already known (inter alia: A.S.Verdini et al., J. Med. Chem. 1991, 34, 3372-3379).

10 Unless otherwise specified, the analytical method used for the determination of the titer or the belonging to the steric series (L or D) of the single aminoacids contained in each tripeptide (excluded those retro-inverted) is based on the method of Noriyuki Nimura et al. in J. Chrom. 352 (1986), 169-177, suitably modified. The IR spectra were recorded in D₂O or KBr on Jasco Mod. Ft/IR 5000 apparatus.

15 The bonds at 3404 cm⁻¹, 3180 cm⁻¹ (stretching N-H peptide and NH imidazole); 3100 cm⁻¹, 2995 cm⁻¹ (stretching NH of NH₃ in NH₂ of lysine and/or salified terminal -NH₂); 1654 cm⁻¹ (stretching CO amide: bond I of the amide); 1565 NH bond bending: band II of the amide); 1239 cm⁻¹ (C-N-stretching + N-H bending: band III of the amide) agree with the peptide structure of the disclosed derivatives whereas the bands at 1469 cm⁻¹ e 1442 cm⁻¹ (ring stretching of the imidazole) agree
20 with the presence of histidine.

25 The FAB-MS (Fast Atom Bombardment Mass Spectroscopy) data were obtained with the VG-70-70 EQ-HF apparatus provided with a standard source, using Xe as gas, glycerol as matrix and temperature of 363°K.
30 All the non retro-inverted samples not containing sarcosine showed a MH⁺ at 340 and those with sarcosine

at 350, in full agreement with the proposed structure.

The purity degree of the single derivatives and their behaviour with respect to different enzymes was determined by RP-HPLC on water 600E apparatus with
 5 Erbasil^R C18S (3 μ), 4x250 mm apparatus, eluent 0.1 M NaClO₄ in phosphoric acid 0.1% v/v, or by capillary electrophoresis on Applied Biosystems 270 A-HT apparatus, KH₂PO₄ buffer, pH = 7.5, at 20 Kv and 30°C.

Abbreviations used:

10	Z	: benzyloxycarbonyl group
	Tos	: tosyl group
	Bzl	: benzyl group
	Boc	: t-butyloxycarbonyl group
	Oct	: n-octyl group
15	D.C.C.	: dicyclohexylcarbodiimide
	HOTB	: 1-hydroxybenzotriazole
	PIP	: piperidine
	DMF	: dimethylformamide
	Pfp	: pentafluorophenyl residue
20	TFA	: trifluoroacetic acid
	Sar	: sarcosine (N-methyl-glycine)
	Bom	: benzyloxymethyl group

Example 1: Gly-His-D Lys (1)

Fmoc-D Lys(Boc)-R, (R: p-benzyloxybenzyl alcohol
 25 resin), after treatment with PIP/DMF to remove the protect the group from α -amino group, added to the pentafluorophenyl activated ester Fmoc-His(Boc)-OPfp. The dimer immobilized on resin, Fmoc-His(Boc)-D Lys(Boc)-R, treated with PIP/DMF, yielded His(Boc)-D
 30 Lys(Boc)-R which was conjugated with Fmoc-Gly-OPfp.

The immobilized and protected tripeptide, Fmoc-

Gly-His(Boc)-D Lys(Boc)-R, after deprotection and separation from the resin to a 90% trifluoroacetic acid in water, was added to a stoichiometric amount (3:1) of HCl and then lyophilized.

5 The derivative Gly-His-D Lys.3HCl was obtained. A sample of the product was transformed in Gly-D His-Lys.ACOH (acetate) by adsorption on Nova Pack HR C18 (Water) HPCL column and elution with 0.2% acetic acid in water.

10 The Gly-His-D Lys Cu (II) complex was prepared dissolving the peptide in water, adding an equimolar amount of monohydrate copper acetate and adjusting the pH with diluted sodium hydrate, under cooling. After centrifugation at low temperature, to make the solution
15 clear, the product was lyophilized.

Aminoacid content (three determinations)

Gly: 1.02 ± 0.01 ; His: 0.98 ± 0.03 ; D Lys: 1.00 ± 0.00

Example 2: Gly-D His-Lys (2)

20 Fmoc-Lys(Boc)-R (R: p-benzyloxybenzyl-alcohol resin), after treatment with PIP/DMF, was conjugated with Fmoc-DHis(Boc)-OH by means of dicyclohexylcarbodiimide (DCC) and hydroxybenzotriazole (HOBt). The reaction with Fmoc-Gly-OPfp and the subsequent deprotection with 90% TFA yielded the
25 trifluoroacetate which, after addition of HCl 3:1 and lyophilization, yielded the tripeptide Gly-D His-Lys.3HCl. The corresponding acetate was obtained as disclosed in Example 1.

30 The copper complex Gly-D His- Lys Cu (II) was prepared as disclosed in Example 1.

Aminoacid content (three determination)

Gly: 1.01 ± 0.02 ; D His: 0.99 ± 0.02 ; Lys: 9.01 ± 0.03

Example 3: Gly-D His-D Lys (3)

Fmoc-D Lys(Boc)-R (R: p-bezyloxybenzyl-alcohol resin) was added, after treatment with PIP/DMF, to
5 Fmoc-D His(Boc)-OH in the presence of DCC/HOBt and then to Fmoc-Gly-Opf. The recovery of the Gly-D His-D Lys.3HCl derivatives was carried out as in the previous examples.

The Gly-D His-D Lys Cu(II) complex was obtained as
10 disclosed in Example 1.

Aminoacid content (three determinations)

Gly: 0.99 ± 0.02 ; D His: 1.04 ± 0.03 ; D Lys: 1.00 ± 0.03

Example 4: (N-methyl)Gly-D His-D Lys (Sar-D His-D Lys) (12)

15 The synthesis of the Sar-D His-D Lys derivative was carried out according to the method disclosed for the derivative Gly-D His-D-Lys except for the conjugation of the dipeptide H-D His (Boc)-D Lys (Boc)-R which was carried out with Fmoc-Sar-OPfp. The
20 preparation of the derivatives Sar-D His-D Lys.3HCl, Sar-D His-D Lys.Ac (acetate) and of the complex Sar-D His-D Lys Cu (II) was carried out as described in the previous examples.

Aminoacid content (three determinations):

25 Gly: 1.03 ± 0.03 ; D His: 1.01 ± 0.02 ; D Lys: 0.97 ± 0.04

Example 5: (N-methyl)Gly-His-D Lys (Sar-His-D Lys: 11) and (N-methyl)Gly-D His-Lys (Sar-D His-Lys: 10)

The two derivatives were synthesized as in the previous examples, using the intermediates H-His(Boc)-D Lys(Boc)-R and H-D His(Boc)-Lys(Boc)-R in the
30 conjugation with Fmoc-Sar-OPfp. The copper complexes

were obtained as in Example 1.

Chemico-physical characteristics:

Aminoacid content (three determinations)

11: Sar: 1.01 ± 0.03 ; His: 0.98 ± 0.02 ; D Lys: 0.99 ± 0.04

5 10: Sar: 0.97 ± 0.02 ; D His: 1.01 ± 0.03 ; Lys: 0.98 ± 0.02

Example 6: Gly-D His-D Lys-O-benzylester (9)

In a suitable solvent (hexane/ethyl acetate) H-D Lys(Z)-O-Bzl and Boc-His(Tos) were conjugated in the presence of D.C.C.. After treatment with NaHCO_3 ,
10 extraction and washing with water, the organic phase was evaporated and the residue crystallized from hexane-ethyl acetate. The crystallized product was dissolved in 50% hydrofluoric acid in dichloromethane and the solvent was then removed. The residue dissolved
15 in hexane/ethyl acetate and in the presence of D.C.C., was conjugated with Boc-Gly. After drying, dissolution in glacial acetic acid and hydrogenation in the presence of Pd/C, the tripeptide was purified by means of cation and anion exchange resins. The eluates were
20 neutralized with acetic acid or HCl. Gly-D His-D Lys-O-Bzl was transformed into the Cu(II) complex as disclosed in Example 1.

Aminoacid content (three determinations):

Gly: 1.01 ± 0.02 ; D His: 0.97 ± 0.05 ; D Lys: 1.00 ± 0.03

25 Example 7: Gly-D His-Lys-O-octylester (4)

Preparation of the derivative (D or L) Lys (Z)-O-R (R: alkyl residue from 1 to 14 straight or branched carbon) according to the patent by L. Pickart (cited patent): H- L (or D) Lys(Z)-OH was treated with an
30 excess of the desired alcohol in anhydrous benzene and traces of p-toluensulfonic acid chloride (or anhydrous

HCl). After refluxing for about 2 hours, the benzene/water azeotrope was continuously distilled for about 12 hours. After this period the mixture was kept at 0°C and the precipitated product was treated with
5 potassium bicarbonate in water and methylene chloride and the organic phase, was evaporated after washing with water and drying on anhydrous magnesium sulphate. The residue was purified by chromatography.

10 The derivative L Lys (Z)-O-Oct, prepared as described above starting from n-octyl alcohol, was dissolved in anhydrous solvent and reacted with Boc-D His (Tos)-OH in the presence of D.C.C. and HOBT. The subsequent treatment with 50% HF in methylene chloride, followed by coupling with Boc-Gly as disclosed in
15 Example 6, optionally in the presence of HOBT, yields Gly-D His-Lys-O-oct, purified by chromatography.

The preparation of corresponding Cu (II) complex was carried out as in Example 1.

Aminoacid content (three determinations)

20 Gly: 0.97 ± 0.02 ; D His: 0.98 ± 0.05 ; D Lys: 0.96 ± 0.05

Example 8: Sar-D His-D Lys-O-benzylester (18)

The derivative D Lys (Z)-O-Oct, prepared as disclosed in Example 7, is reacted with Boc- D His(Tos)-OH in the presence of D.C.C. and HOBT in the
25 same conditions above reported; the obtained derivative was condensed with Boc-Sar with D.C.C./HOBT and the crude product, dissolved in glacial acetic acid, was hydrogenated with Pd/C and the final product purified by chromatography.

30 The complex Sar-D His-D Lys-O-benzylester Cu (II) was obtained as disclosed in Example 1.

Aminoacid content (three determinations)

Sar: 0.96 ± 0.04 ; D His: 0.98 ± 0.02 ; D Lys: 0.97 ± 0.05

Example 9: Gly-gem His-(R,S)m-Lys (19)

a) Boc-Gly-g-His(Bom)-H

- 5 1.2 g (3.05 mmol) of Boc-His(Bom)-OH (Bachem) were dissolved in 20 ml of dichloromethane containing 3.05 mmol pyridine and 3.05 mol pentafluorophenol; 7.625 mmol of dicyclohexylcarbodiimide (D.C.C.) were added to this solution, cooled at 0°C. After
- 10 3 h the reaction mixture was filtered, dried and the residue, repeatedly washed with petroleum ether, was suspended in 10 ml of tetrahydrofuran, 2.6 ml of 25% ammonia were added, under stirring, to this suspension, obtaining a yellow solution;
- 15 this was evaporated and the residue dissolved in 100 ml of chloroform. The chloroform solution was washed with 10 ml of water, 20 ml of 5% NaHCO_3 , then again with NaCl saturated water until neutrality of the washing liquid. The residue
- 20 obtained from the chloroform solution was suspended in 12 ml of HCl 3.2 M and stirred at 60°C for 20 min; the solution obtained, was adjusted to pH 7.5 with NaHCO_3 (3.6 g) after cooling. A residue was obtained from this
- 25 evaporated solution, which was thoroughly dehydrated by means of repeated additions of benzene-methanol 6:4 azeotropic mixture, and then the inorganic salts were removed by repeated chloroform extractions.
- 30 The obtained amide (1.787 mmol) was dissolved in 13 ml of dichloromethane together with an

equivalent amount of triethylamine.

After the addition of 1.787 mmol of Boc-Gly-OPfp (obtained starting from Boc-Gly, Bachem, by activating the carboxy group with pentafluorophenol according to the method above disclosed for histidine) the solution was left under stirring at room temperature for 2 h.

The reaction mixture was evaporated, dissolved in acetonitrile and evaporated again, repeating this operation many times. The residue was finally dissolved with the minimum amount of CHCl_3 and purified on 70-230 mesh Merck silica gel column, 20 g, using CHCl_3 -MeOH 9:1 as eluent.

600 mg of pure Boc-Gly-His(Bom)- NH_2 were thus obtained. The dipeptide obtained in form of amide, as above disclosed (1.386 mol) has been dissolved in 6 ml of CH_3CN and 1.5 ml of H_2O were then added; the bis-trifluoroacetoxy-iodobenzene reagent has been added thereto (820 mg; 2.087 mmol) and then 220 μl of pyridine.

The reaction mixture was then stirred at room temperature for 1 h and 526 mg of solid NaHCO_3 were then added and the mixture was stirred for 10 additional minutes. The mixture was finally evaporated and, after drying by means of repeated evaporations with benzene-methanol azeotropic mixture, the residue was purified on Merck 70-230 mesh silica gel column, 55 g and multiple-step elution. The used mobile phases were respectively: 1, CHCl_3 -MeOH 7:3, 2), CHCl_3 -MeOH 10:1, 3), CHCl_3 -MeOH 7:1, 4), CHCl_3 -MeOH 6:1.

505 mg of Boc-Gly-g-His(Bom)H were thus obtained.

b) 2,2-Dimethyl-5-(N-trifluoroacetyl-butyl)-1,3-dioxan-4,6-dione

5 4-NH₂-butyrraldehyde diethylacetal (3.7 ml, 21.4 mmol) was dissolved, together with 2.62 g of diethylaminopyridine in 40 ml of CHCl₃ and the solution cooled at 0°C; 3 ml of trifluoroacetic anhydride were added dropwise, under stirring. Stirring was continued for 30 minutes, maintaining
10 the temperature between 5 and 10°C, and then again for 2 additional h at room temperature. The suspension was filtered, the filtrate washed with 10 ml of NaHCO₃. After one night at room temperature, this solution was saturated with NaCl
15 and extracted with dichloromethane (1x30 ml, 1x20 ml and 4x10 ml). The residue of the pooled and concentrated organic phases was a yellow oil, about 3 g, which was directly used in the subsequent step.
20 8.7 ml of a cyanoborohydride solution (1M in THF) were evaporated under vacuum and the residue dissolved in 5 ml of DMF. The Meldrum acid (2,2-dimethyl-1,3-dioxan-4,6-dione) 1.79 g, 12.4 mol) was also added to this solution. This solution was
25 then added to the oil obtained in the first step, cooling at 0-5°C to avoid an exothermic reaction and the mixture was left at room temperature for 2 h.
The reaction mixture was then diluted with 40 ml
30 of cold H₂O: a white precipitate was formed and the pH was adjusted to 4 by careful adding conc.

HCl. This suspension was stirred, always at 0-5°C, for 1 h and then filtered; the product, washed on the filter with H₂O and then with ether, and thereafter thoroughly dried on P₂O₅, had m.p. 129-130°C, with final yield of 40%.

5 c) 2,2-Dimethyl-5-(N-Boc-butyl)-1,3-dioxan-4,6-dione
1.17 g (3.77 mol) of N-trifluoroacetylbutyl derivative (prepared in b) were suspended in 8 ml dioxane. 1.9 ml of 5 N NaOH were added and the
10 resulting clear solution was left under stirring at room temperature for 4 h. After cooling to 5°C, di-tert-butylidicarbonate (1.6 ml, 7.54 mmol) was added dropwise. The reaction mixture was kept at room temperature for 12 h adjusting the pH to 8,
15 if necessary. At the end, the solution was diluted with 10 ml water and filtered. The filtrate was evaporated under vacuum to halve its volume, diluted again, and the same process was repeated until elimination of dioxane.
20 The pH was adjusted to 5 with 1M KHSO₄ and the solution extracted with CHCl₃ (1x50 and 2x20 ml). After evaporation of solvent, the oily residue was purified on a silica gel column (50 g) with CHCl₃/EtOAc 5:1.

25 The pure product was obtained with 74% yield.

d) Boc-Gly-g-His(Bom)-m-Lys(Tfa)-OH
1.012 g (3.25 mol) of 2,2-dimethyl-5-(N-trifluoroacetylbutyl)-1,3-dioxan-4,6-dione were
30 mixed with 598 mg of pentafluorophenol and heated to the melting temperature of the mixture (about 110°C). The fusion mixture was stirred for 3 h at

this temperature and then subjected to vacuum by water pump for 15 more minutes. The residue was dissolved in 10 ml of dichloromethane and 1.311 g of Boc-Gly-g-His(Bom)-H and 850 μ l of triethylamine were added. After stirring overnight at room temperature, the reaction mixture was purified by preparative HPLC on Deltapack column C18-100 A Waters, 15 μ , eluted with a gradient from solution A (H_2O + 0.1% Tfa) + Sol. B ($\text{CH}_3\text{CN}-\text{H}_2\text{O}$, 80:20 + 0.09% Tfa) 50:50, up to 0-100 in 20 minutes. Flow 12 ml/min.

e) Boc-Gly-g-His(Bom)-m-Lys(Tfa)-OPh

528.2 mg (1.7 mmol) of 2,2-dimethyl-5-(N-trifluoroacetylbutyl)-1,3-dioxan-4,6-dione were mixed with 159.7 mg of phenol and heated to the melting temperature of the mixture (110°C), under stirring for 25 h and then 15 minutes under vacuum. After cooling the oil was dissolved in 4 ml of NaHCO_3 saturated solution which was washed with CHCl_3 (1x10 and 2x5 ml). The organic phases, washed again with 5 ml of H_2O and 5 ml of NaCl saturated solution, dried on Na_2SO_4 and evaporated, gave a white solid residue consisting of pure product with 74% yield. 1.677 g (4.83 mol) of the previously obtained phenylester, 1.95 g (4.83 mol) of Boc-Gly-g-His(Bom)-H, 0.390 μ l of pyridine, 783 mg (5.705 mol) of hydroxybenzotriazole were dissolved in 40 ml of dichloromethane and the solution cooled to 0°C; under stirring, 1.315 of DCC were then added and the reaction was continued for 3 h at room

temperature. At the end the suspension was filtered, the filtrate diluted with 400 ml of dichloromethane, washed with 50 ml of 10% Na_2CO_3 solution and then with different portions of H_2O and NaCl saturated solution. The solvent, dried and concentrated, gave 2 g of the completely protected tripeptide as yellow solid, 57% yield.

f) Boc-Gly-g-His(Bom)-m-Lys (Boc)-OH

770 mg (1.91 mmol) of Boc-Gly-g-His(Bom)-H were suspended in 15 ml of CH_2Cl_2 under stirring in Argon atmosphere and 1.2 g (5.91 mol) of N,O-bis-silylacetamide (BSA) were added to the suspension. A clear solution was obtained which was refluxed for 6 h and thereafter cooled to room temperature. 542 mg (1.72 mmol) of 2,2-dimethyl-5-(N-Boc-butyl)-1,3-dioxan-4,6-dione were then added and stirred overnight (about 16 h).

The reaction mixture was finally evaporated and the ter-butyloxycarbonyl group was directly removed as disclosed in Example 7.

g) Removal of Boc groups from Gly and Lys

The residue from the previous reaction (see Example 6) was treated with 29 ml of trifluoroacetic acid and stirred at room temperature for 20 minutes, then evaporated, dissolved again in 10 ml of CH_3CN and purified on preparative HPLC column:

Column: Deltapack 19x300 mm, 15 micron, Waters.

Mobile phase: A, 0.1% Tfa in H_2O ; B, 20% CH_3CN in H_2O + 0.09 Tfa, linear gradient of B from 0 to 20% in 20 min.

Flow: 19 ml/min; injection volume: 1 ml

The recovered residue consisted of 586 mg of Gly-g-His(Bom)-m-Lys-OH in trifluoroacetate form, 95% pure. Yield 42.5%.

5 h) Removal of the benzyloxymethyl group from the gem-histidine residue

586 mg (0.73 mmol) of Gly-g-His(Bom)-m-Lys-OH and 4.74 g of thioanisole (38.16 mmol) were dissolved in 24 ml of TFA in Argon atmosphere. After cooling
10 to 1-2°C 8.48 g (38.16 mmol) of triflate (trimethyl-silyl-trifluoromethanesulfonate) were added and the mixture was stirred for 30 min. 36 ml of ethyl ether were then added: a white precipitate, separated by centrifugation, was
15 obtained. The residue was washed by decantation with other 20 ml of ether three times. The residue was finally dissolved in 21 ml of 5% NH_4OH containing 1.18 g of NH_4F , stirred for 1 h and the pH adjusted to 5 with AcOH 5N.

20 Chromatographic conditions:

Column: Novopack 19x300 mm, HRC18, A°, 6 micron, Waters

Mobile phase: 0.2% AcOH H_2O

Flow: 12 ml/min; 200 μl injection

25 Each fraction containing the product was pooled and concentrated, the residue dissolved with 1 ml of H_2O and evaporated again, repeating this operation many times, so as to remove the excess acetic acid from the residue. At the end, after
30 lyophilization, 86 mg of pure peptide were obtained corresponding to the following structure:

H-Gly-g-His-m-Lys-OH.AcOH.2.5.H₂O.

Elemental analysis: Calc. %: C 43.14, H 7.41, N 18.87

Trov. %: C 42.82, H 7.18, N 18.83

¹H-NMR, 10% in D₂O, ppm: 8.38, 7.31 (s, CH imid.); 5.95
5 (t, CH g-His); 3.90 (s, CH₂ Gly); 3.38-2.98 (m;
CH₂ g-His; CH₂NH₂m-Lys; CH m-Lys); 2.04 (s,
CH₃COO⁻); 1.78 (m, α and β CH₂ m-Lys); 1.38 (q, γ
CH₂ m-Lys).

i) Removal of all the protective groups of the
10 tripeptide Boc-Gly-g-His(Boc)-m-Lys(Tfa)OPh

125 mg (0.17 mmol) of the fully protected peptide,
obtained as described in e), were dissolved in 0.5
ml of MeOH + 0.17 ml of NaOH 5N and stirred for 4
h at room temperature. Sodium hydroxide was then
15 neutralized with 3N HCl and methanol was
evaporated. The residue was diluted in 2 ml of H₂O
and washed with CHCl₃ (2x0.5 ml). The aqueous
phase was evaporated under vacuum and H₂O traces
were removed by repeated evaporations with
20 azeotropic mixture. The residue (232 mg) was
directly used in the subsequent step.

The previous residue was suspended with 200 mg of
NaI in 2 ml of CH₃CN, under stirring and Argon
atmosphere. 0.17 ml of trimethylsilyl chloride
25 were added to the suspension which was kept at
80°C for 3 h. 130 mg of NaI and 0.11 ml of TMSiCl
were then added and left in the same conditions
for three hours. After cooling to room
temperature, 270 mg of sodium thiosulfate in 4 ml
30 of H₂O were added to the mixture; a clear solution
was obtained having pH 4 which was then

adjusted to 7 with NaOH. The formed precipitate was removed by filtration and the dissolved product exactly purified as in Example 8.

5 The pure peptide H-Gly-g-His-m-Lys-OH monoacetate had the same characteristics as those obtained with a different synthetic method as disclosed in h).

1) Preparation of copper complex of the tripeptide G-gH-mL

10 32.3 mg (0.095 mmol) of G-gH-mL were dissolved in 0.3 ml of H₂O and 0.7 ml of an aqueous solution containing 22.6 mg of Cu(CH₃COO)₂.H₂O (0.113 mmol) were added to this solution. The solution was neutralized with 0.1N (2.4 ml) NaOH, left
15 overnight in the refrigerator (4°C) and then centrifuged and decanted.

The supernatant was eluted through a Sephadex G-10 column, eluted with distilled water and lyophilized, to give 34.7 mg of blue powder,
20 consisting of the G-gH-mL-Cu.

Example 10: H-gem Gly-D His-(R,S) m Lys OH (22)

The product was obtained starting from the monophenyl ester of the 2-N-trifluoroacetylbutylmalonic acid (described in Example 9) and H-D His(Bom)-
25 O-tBu in the presence of HOBT/DCC. After acid treatment, tBu-O-D His(Bom)-(R,S) mLys(TFA)-O-Ph so obtained was transformed in HO- D His(Bom)-(R,S) mLys(TFA)-O- Ph which was reacted with glycine in the presence of HOBT and DCC.

30 The amide residue of the derivative H₂N-CO-CH₂-NH-CO-D His(Bom)-(R,S) mLys(TFA)-OPh was transformed in

amino group with TIB in DMF. The removal of the protective groups, carried out with alkali first and then with trimethylsilyliodide, yielded the derivative H-gem Gly-D His-(R,S) mLys-OH.

5 The NMR spectrum confirms the expected structure.

 Similarly, H-gem Gly-(R,S) m-His-L Lys-OH and H-gem Gly-(r,s) m His-D Lys-OH were prepared.

Example 11: biological activities

a) wound healing

10 First method:

 Dunkin-Hartley guinea pigs were anesthetized and five square wounds (7x7 mm) were excised on the mid-back, completely removing the dermal surface, after shaving and cleaning the dorsal skin (F. Buffoni et al. 15 - Pharmacol. Res. 25, suppl. 2, 332, 1992). The wounds, both in control and treated animals, were allowed to heal. Four, eight and eleven days after surgery, five animals/group were sacrificed and newly formed tissue was dissected and analyzed by histological and 20 biochemical methods.

 The following biochemical parameters were determined: hydroxyproline production (index of collagen formation) (J.F. Woessner, Arch. Biochem. Biophys. 93, 440, 1961); protein content (O.H. Lowry et 25 al., J. Biol. Chem. 193, 265, 1951); DNA content (C. Labarca et al., Anal. Biochem. 102, 344, 1980).

 Histology was performed after staining with hematoxylin/eosin.

 Experimental groups (15 animals/group): 1) 30 controls, receiving 20 µl of distilled water; 2) animals treated with 10 µg of the Cu (II) complexes

described in Table 1 dissolved in 20 μ l of distilled water; 3) animals treated with 10 μ g of GHL-Cu(II) complex in 20 μ l of distilled water. Administration route: water solutions were applied onto the wound.

TABLE 1

Parameter:	DNA ⁽¹⁾			Proteins ⁽¹⁾			Hydroxyproline ⁽¹⁾		
	4°	8°	11°	4°	8°	11°	4°	8°	11°
analyzed at day:									
Substance:									
Controls	11.6	1.7	3.5	334	155	155	21.5	7.8	12.0
Gly-His-Lys (Cu)	16.3	2.4	3.9	375	176	184	21.0	6.9	14.3
<u>2</u> (Cu)	15.6	3.2	2.6	410	189	115	22.1	9.3	12.0
<u>3</u> (Cu)	16.1	3.6	2.0	415	191	130	21.7	9.4	11.9
<u>9</u> (Cu)	15.8	3.1	2.1	405	140	140	21.8	9.1	11.8
<u>12</u> (Cu)	15.8	3.5	1.8	420	196	155	22.3	9.4	11.9
<u>17</u> (Cu)	16.1	3.6	2.0	426	201	148	22.4	9.3	12.0
<u>19</u> (Cu)	15.0	3.4	2.2	425	200	129	22.5	9.7	11.8
<u>22</u> (Cu)	16.3	3.5	2.2	412	199	142	22.2	9.7	12.0

⁽¹⁾ mg/g of fresh tissue

The pieces of tissue removed from the wounds on the 4th day contain both the regenerated tissue and the scab (mainly cluster of dead cells) and thus the three analyzed parameters presented higher values than those at the 8th and the 11th day. However, the results obtained from the animals treated with the products hereinbefore described show higher chemotaxis and/or increased production of extracellular matrix in comparison to those obtained from animals untreated or treated with GHL-Cu(II) .

The comparison of the three parameter values obtained at the 8th and 11th day clearly shows that in the animals treated with the compounds described in the present patent application the skin was already well restored at the 8th day whereas in the animals treated with GHL-Cu(II) the same degree of healing was reached at the 11th day. This indicates that the compounds reduced the time of wound healing with respect to the controls and the GHL-Cu(II) treated.

At the histological analysis, the tissues from the animals treated with the compounds hereinabove described turned out well organized.

Second method:

Hairs were removed from the dorsal skin of Wistar rats (approximate weight 220 g) and two round wounds (\emptyset 10 mm) were induced by punch under anesthesia. Ten min after the excision, the wounds were treated with 20 μl of distilled water alone (control group) or containing 10 μg of the products to be tested (treated groups), then covered with a surgical gauze. The gauze was kept distant from the wound bed by a teflon ring with

appropriate diameter fixed with an adhesive bandage. The treatment was repeated for three consecutive days. On the 5th, 8th, and 12th day the percentage rate of wound healing was evaluated assuming:

- 5 100% : total healing
 0% : absence of healing

 The experimental groups consisted of 6 animals/group. Administration route: water solution applied onto the wound. The results, shown in Table 2,
10 indicate that the compounds described in the present application reduced the time of wound healing with respect to the native product. Therefore the histological analysis shows a complete restitutio ad
15 integrum of the tissues and an angiogenetic effect similar to those obtained with physiological timing.

TABLE 2

Product	% of healing			
	at day:			
	5°	8°	12°	
Controls	19	60	100	
Gly-His-Lys (Cu)	49	70	98	
<u>2</u> (Cu)	65	98	100	
<u>3</u> (Cu)	68	100	-	
<u>4</u> (Cu)	70	100	-	
<u>12</u> (Cu)	63	96	100	
<u>17</u> (Cu)	65	96	100	
<u>19</u> (Cu)	70	100	-	
<u>22</u> (Cu)	75	100	-	

b) Superoxide dismutase activity

The superoxide dismutase activity of the products hereinafter described was determined according to C. Beauchamp et al. (Anal. Biochem. 44, 276, 1971). The method is based on the appearance of coloured species with a maximum of absorption at 560 nm by NBT (nitroblue tetrazolium) in the presence of superoxide ion. The reduction in the intensity of absorption at 560 nm in the presence of the product under investigation determines its SOD-like activity. This activity is expressed in units defined as the concentration of product, in nmoles/ml, able to induce 50% of the maximum inhibition. The results, reported in Table 3, show that the tested derivatives presented an activity equal to GHL-Cu(II) activity.

TABLE 3

Product	Units of activity (nmol/ml)
Gly-His-Lys (Cu)	3.3 ± 0.2
<u>2</u> (Cu)	3.1 ± 0.2
<u>3</u> (Cu)	2.8 ± 0.1
<u>9</u> (Cu)	2.9 ± 0.2
<u>12</u> (Cu)	3.3 ± 0.2
<u>17</u> (Cu)	3.3 ± 0.1
<u>19</u> (Cu)	3.4 ± 0.1
<u>22</u> (Cu)	3.1 ± 0.2

c) Anti-thromboxane activity

The activity of the products under examination was determined in rabbit PRP (platelet rich plasma), according to N. Lad et al. (Br. J. Pharmacol. 69, 3, 1980). TxB2 final titration was performed with the
5 Thromboxane B2 125-I Assay System (Amersham Life Science, U.K.).

The results, reported in Table 4, show that the investigated products have an activity equivalent or
10 superior to GHL-Cu(II) one.

TABLE 4

5

Product (1 µg/ml)	% of inhibition of the TxB ₂ production
Gly-His-Lys (Cu)	83 ± 3
<u>2</u> (Cu)	86 ± 5
<u>4</u> (Cu)	89 ± 4
<u>12</u> (Cu)	92 ± 5
<u>17</u> (Cu)	88 ± 2
<u>19</u> (Cu)	91 ± 3
<u>22</u> (Cu)	90 ± 3

Example 12: resistance to peptidases

The stability of the derivatives described in Table 5 was tested in presence of mitochondrial leucineaminopeptidase (aminopeptidase M), B
5 carboxypeptidase and human plasma from healthy volunteers. The residual quantity of each one of the tested products was determined at different experimental times by HPLC using the above described conditions. The results obtained after 5 and 35 min of
10 exposure to the enzymatic action (phosphate buffer pH = 7.4 at 37°C for both pure enzymes and undiluted plasma) are reported in Table 5. The data show a much higher resistance of the tested products to the enzyme, in comparison to native GHL.

TABLE 5

Product	Aminopeptidase M (0.06 U/ml); % starting peptide at:		Carboxypeptidase B (1 U/ml); % starting peptide at:		Human plasma % starting peptide at:	
	5'	35'	5'	35'	5'	35'
Gly-His-Lys (Cu)						
<u>2</u> (Cu)	30	(1) N.D.	55	(1) N.D.	40	(1) N.D.
<u>3</u> (Cu)	80	75	80	65	-	-
<u>9</u> (Cu)	92	70	90	83	-	-
<u>9</u> (Cu)	95	78	92	75	-	-
<u>12</u> (Cu)	93	82	89	78	85	70
<u>17</u> (Cu)	91	86	95	86	87	68
<u>19</u> (Cu)	87	(2) 59	98	(3) 98	90	60
<u>22</u> (Cu)	95	95	98	98	95	80

(1) No longer analytically detectable at $t > 20'$ (2) No longer analytically detectable at $t > 150'$ (3) This value remains constant up to $t > 120'$

This increased resistance accounts for the greater biological activities shown in vivo by these compounds.

No significant difference between free peptides and peptide copper complexes has been detected as far
5 as their biological activity.

CLAIMS

1. Compounds of general formula (I)



5 wherein:

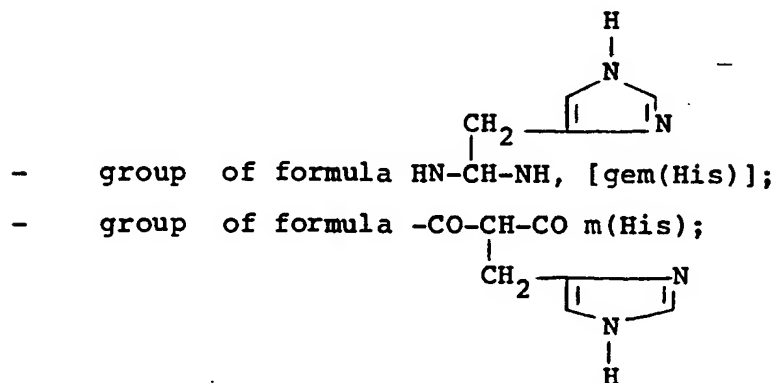
Gly is one of the following residues:

- glycine;
- sarcosine;
- group of formula $\text{NH}_2\text{-CH}_2\text{NH-}$, [gem(Gly)];

10 His is one of the following residues:

- L-histidine;
- D-histidine;

15

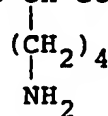


20

Lys is one of the following residues:

- L-lysine;
- D-lysine;
- group of formula -CO-CH-CO-(m-Lys)

25



30

R is hydrogen, straight or branched $\text{C}_1\text{-C}_{14}$ alkyl, $\text{C}_6\text{-C}_{14}$ aryl or aralkyl residue, with the proviso that Gly, His and Lys cannot be contemporaneously the natural amino acids glycine, L-histidine and L-lysine, and pharmaceutically acceptable salts and the copper

complexes thereof.

2. Compounds according to claim 1 wherein His or Lys is a residue of the corresponding D aminoacid and R is H.

5 3. Compounds according to claim 1 wherein His or Lys is a residue of the corresponding D aminoacid and R is different from H.

4. Compounds according to claim 1 wherein His or Lys are both residue of the corresponding D aminoacids and
10 R is H.

5. Compounds according to claim 1 wherein His or Lys are both residue of the corresponding D aminoacids and R is different from H.

6. Compounds according to claim 1 wherein Gly is the
15 sarcosine residue whereas His, Lys and R are as defined in any one of the above points 1-4.

7. Compounds according to claim 1 wherein Gly is a gem-diaminal residue as above defined [gem(Gly)] whereas one of the His and Lys residue is a residue
20 m(His) or m(Lys) as above defined whereas the other is a residue of the L or D series and R is hydrogen.

8. Compounds according to claim 1 wherein Gly is a gem-diaminal residue as above defined [gem(Gly)] whereas one of the His and Lys residue is a residue
25 m(His) or m(Lys) as above defined whereas the other is a residue of the L or D series and R is different from hydrogen.

9. Compounds according to claim 1 wherein Gly is glycine, His is gem-His and Lys is m(Lys) as above
30 defined and R is hydrogen.

10. Compounds according to claim 1 wherein Gly is

glycine, His is gem-His and Lys is m(Lys) as above defined and R is different from hydrogen.

11. Use of the compounds of claims 1-10 as therapeutic agents.

5 12. Use of the compounds of claims 1-10 for the preparation of drugs useful in the treatment of ulcers, tissue damages of different etiology, autoimmune degenerative pathological conditions.

10 13. Pharmaceutical compositions containing a therapeutically effective amount of a compound of claims 1-10 as the active principle.

INTERNATIONAL SEARCH REPORT

Internat Application No

PCT/EP 93/02004

A. CLASSIFICATION OF SUBJECT MATTER

IPC 5 C07K5/08 C07D233/64 A61K37/02 A61K31/415

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 5 C07K C07D A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	EP,A,0 288 278 (PROCYTE CORPORATION) 26 October 1988 see the whole document ---	1-13
Y	B.WEINSTEIN 'CHEMISTRY AND BIOCHEMISTRY OF AMINO ACIDS, PEPTIDES AND PROTEINS; Vol. 1.7' 1983, MARCEL DEKKER, INC., NEW YORK Chapter 5; A.F.SPATOLA, "PEPTIDE BACKBONE MODIFICATIONS", pages 268-357 ---	1-13
A	EP,A,0 199 379 (ENIRICERCH) 29 October 1986 see the whole document --- -/--	1-13

☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

* Special categories of cited documents :

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

T later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

X document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

Y document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

& document member of the same patent family

Date of the actual completion of the international search

14 December 1993

Date of mailing of the international search report

9. 01. 94

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Groenendijk, M

INTERNATIONAL SEARCH REPORT

Internati Application No
PCT/EP 93/02004

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	<p>INTERNATIONAL JOURNAL OF PEPTIDE AND PROTEIN RESEARCH vol. 41, no. 6 , June 1993 , COPENHAGEN DK pages 561 - 566 A.DALPOZZO ET AL 'H-Gly-His((NHCO)Lys-OH, patially modified retro-inverso analogue of the growth factor GHK with enhanced enzymatic stability' see the whole document -----</p>	1,9-13

INTERNATIONAL SEARCH REPORT

International application No.

PCT/EP 93/02004

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark : Although claim 11 is directed to a method of treatment of (diagnostic method practised on) the human/animal body the search has been carried out and based on the alleged effects of the compound/ composition.
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Internati

Application No

PCT/EP 93/02004

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A-0288278	26-10-88	US-A- 4877770	31-10-89
EP-A-0199379	29-10-86	JP-A- 61233665	17-10-86
		US-A- 4748155	31-05-88